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Biopsy markers of airway inflammation and remodelling

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Bronchial inflammation is a consistent feature of asthma and its chronicity probably determines disease progression. Clinical evaluation of drugs with potential disease-modifying activity requires measurement of their effects on the inflammatory and remodelling process using a variety of techniques including bronchial biopsy, and analysis of sputum, broncholaveolar lavage, blood, urine and exhaled air.

Markers of the key components of the inflammatory process, such as the number and activation of T-cells, the number of mast cells, cytokine and chemokine release or gene expression, and eosinophil and neutrophil recruitment, can be determined in biopsy samples. Biopsies also allow assessment of the integrity and structure of the airway epithelium, the thickness of the reticular basement membrane and the numbers and ultrastructure of contractile cells. These and other markers may allow differentiation between subtypes of asthma patient according to atopic status and will help to distinguish asthma from chronic obstructive pulmonary disease.

Airway remodelling may be a consequence of chronic bronchial inflammation and is a characteristic of chronic asthma, particularly in severe asthma and when there is progressive decline in lung function. There are changes in the surface epithelium, reticular basement membrane, bronchial smooth muscle, blood vessels and mucous glands. Reliable markers of remodelling need to be identified to improve our ability to evaluate chronic asthma therapy.

Key words: asthma; inflammation; remodelling; biopsy.

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Introduction

Eosinophilic inflammation of the airways is common in patients with mild and moderately severe asthma and is considered to be fundamental to the functional changes that characterize the disease (1–3). Persistence of a chronic inflammatory response in asthmatic patients is thought to initiate the progressive structural change known as 'airway remodelling', which is probably a major determinant of increasing disease severity (4,5).

Consequently, pharmacotherapy which is demonstrated to have an anti-inflammatory property may prevent or slow the progress of asthma, rather than simply alleviating symptoms. As none of the clinical measures used to diagnose asthma can give precise and specific information about inflammation, more direct assessment is necessary, using techniques such as bronchial biopsy, sputum analysis, bronchoalveolar lavage (BAL) and analysis of exhaled air. By measuring changes in selected markers, for example recruitment and function of a variety of inflammatory cell types, production of inflammatory mediators and expression of genes that control synthesis of pro-inflammatory proteins or influence the remodelling process, the underlying disease process can be monitored (6,7).

This overview considers appropriate markers for evaluation in bronchial biopsies where acute and chronic inflammation can be assessed *in situ* (8). Some of these markers may be useful in the future to evaluate the process of airway remodelling. It should be recognized, however, that interpretation of results from bronchial biopsy analysis poses challenges, not least because of the large degree of variability that exists between and within patients.

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Variation in asthmatic phenotype

One factor associated with asthma that can be measured simply is atopy. The presence of atopy is associated with inflammation, whether or not the patient presents with asthma. In one study, biopsies from atopic non-asthmatic patients had more activated ($EG2^+$) eosinophils and a thicker reticular basement membrane (RBM) than those from healthy controls, but these changes were even more pronounced in atopic patients with asthma (9).

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Eosinophilia, whilst variable, is a fundamental feature of asthma (10–13) and the associated increase in expression of the cytokines interleukin (IL)-4 and IL-13 (which promote selective vascular adhesion of these cells) appear to be of equal importance in atopic and non-atopic asthma (14). Expression of eosinophil chemokines such as eotaxin, monocyte chemoattractant protein-3 and RANTES, and of IL-4 and IL-5, the T helper-2 (Th_2) cytokines, appears to be similar in the two patient sub-types (15,16); however, expression of granulocyte macrophage colony stimulating factor (GM-CSF), which has been shown to be predominantly associated with $CD68^+$ macrophages, appears to be greater in non-atopic than atopic patients (17).

In non-atopic and severe intractable asthma, neutrophil numbers appear to be significantly increased (18,19). The significance of this observation has yet to be clarified; in some patients, it may be a functional response to treatment with corticosteroids which, *in vitro*, have been shown to down-regulate apoptosis of neutrophils and thus promote their longevity (20).

The amount of the matrix glycoprotein, tenascin, in the RBM is greater in chronic and seasonal asthmatic patients than in healthy controls, and this elevation in levels appears to be more pronounced in patients with chronic disease (21). Epithelial integrity is reduced in atopic asthmatic patients compared to healthy controls and has been shown to be correlated with an increase in airway reactivity (22); significant correlations have also been observed between epithelial integrity in atopic patients and the number of $EG2^+$ eosinophils and $CD4^+$ T-cells (18).

Selection of markers of clinical inflammation

Many inflammatory markers can be followed in studies of asthmatic subjects; selected examples are listed in Table 1. In order to monitor inflammatory changes in atopic asthma, a marker of eosinophils is one obvious choice. EG1 and EG2, markers of eosinophilic cationic protein (ECP), are claimed to represent total and activated eosinophil counts, respectively. However, the validity of this depends on several factors including the way in which the cells are fixed and processed (23). Several studies have shown that $EG2^+$ cells may outnumber $EG1^+$ cells; weak EG2 positivity may be found in neutrophils, and macrophages may be rendered EG2-positive by ingesting apoptotic eosinophils. These antibodies can be applied to biopsy sections, or measurements of $EG2^+$ cells in fluid compartments can be obtained using flow cytometry. As eosinophils tend to cluster, their tissue distribution is not uniform, and they are frequently absent from single biopsy sections; several sections need to be assessed before a value representative of the biopsy is obtained (24,25). If biopsy is not practicable, eosinophil markers, e.g. ECF, can also be determined in blood, BAL or sputum. In biopsy,

TABLE 1. Markers of inflammation: selected examples

Tissue	Cell	Marker
Biopsies	Eosinophil	EG2, EPO
	Neutrophil	MPO, HNL
	T lymphocyte	CD3, CD4, CD8
	Activated T lymphocyte	CD25
	Macrophage	CD68
	Regulatory cytokines	IL-4, IL-5
	Chemokines	IL-8/eotaxin
	Structural changes	Thickened RBM
		Myofibroblast
		Goblet cell
Blood (serum or plasma)	Eosinophil	ECP,EPO
		Numbers in blood
	Neutrophil	MPO, HNL
	Cell surface markers	ICAM-1/VCAM-1
Sputum	Eosinophil	ECP,EPO
	Neutrophil	HNL
Urine	Eosinophil	EPX (EDN)
Expired air		Nitric oxide
		Hydrogen peroxide
		Carbon monoxide
		Ethane

ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; EPX, eosinophil protein-X, also known as eosinophil-derived neurotoxin (EDN); HNL, human neutrophil lipocalin; ICAM-1, intracellular cell adhesion molecule-1; IL, interleukin; MPO, myeloperoxidase; RBM, reticular basement membranel; VCAM, vascular cell adhesion molecule-1.

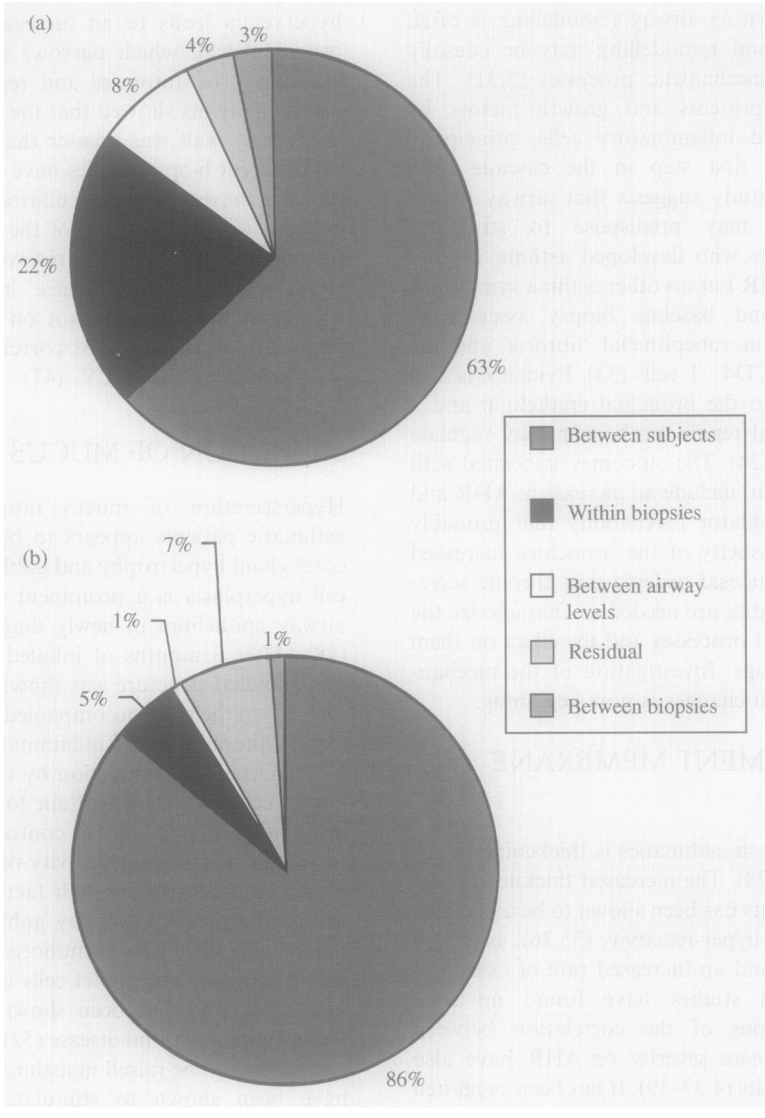

FIG 1. Contribution of inter-subject variability to the total variability of $CD45^+$ cell numbers in bronchial biopsies taken from (a) asthma patients and (b) healthy individuals (unpublished data).

T-lymphocytes and their subsets and cells expressing the gene or protein for selected cytokines, e.g. IL-4, IL-5 or IL-8, can be counted, and structural changes can be measured such as epithelial integrity (22,26), thickness of the RBM and its content of tenascin, laminin and 'collagen' subtypes (21,27,28). Neutrophil markers are appropriate for non-atopic asthma, atopic asthma following allergen challenge or in severe asthma. Levels of myeloperoxidase (MPO) are twice as high in the plasma of non-atopic asthmatic patients than in atopic patients and healthy controls (unpublished data); this enzyme can also be determined in bronchoalveolar lavage fluid. In addition, human neutrophil lipocalin (HNL) is a very stable and specific neutrophil marker which can be measured in induced sputum samples or used as a neutrophil marker in tissues (29).

By selecting one key marker for each cell type, the total number of possible variables monitored can be reduced. Importantly, the variability between patients in bronchial biopsy studies is a major contributor to total variability in the perceived levels of a given marker, as illustrated in Fig. 1 for $CD45^+$ T-cells 'unpublished data'. In studies where the variability between patients is expected to be high, reinforcement of results may be achieved by following two or more linked markers to confirm that a particular mechanistic 'axis' is affected. An example of such an axis would be the numbers of TH_2 cells with expression of one of the associated cytokines IL-4 or IL-5, and the numbers of eosinophils (30). Ideally, the axis chosen should be clearly linked with clinical outcomes, e.g. an improvement in airway hyper-responsiveness.

Airway remodelling

When inflammation in the airways of asthmatic patients becomes chronic, a process of inappropriate progressive

structural changes known as airway remodelling is often present; inflammation and remodelling may be causally linked or be separate mechanistic processes (5,31). The production of matrix proteins and growth factors by populations of activated inflammatory cells, principally eosinophils, may be a first step in the cascade (32). Alternatively, a recent study suggests that airway hyperresponsiveness (AHR) may predispose to structural changes; in four patients who developed asthma 2 years after presenting with AHR but no other asthma symptoms, comparison of final and baseline biopsy assessments showed early increases in subepithelial 'fibrosis' and the number of $CD25^+$ and $CD4^+$ T cells (33). Evidence is also emerging that damage to the bronchial epithelium and a disruption of the normal repair mechanism may regulate subsequent remodelling (34). The outcomes associated with remodelling are thought to include an increase in AHR and a reduction in bronchodilator reversibility that probably results from reduced elasticity of the bronchus, increased smooth muscle mass, mucosal oedema and chronic secretion of mucus. More studies are needed to characterize the sequence of these distinct processes and the effect on them of disease-modifying drugs. Investigation of the mechanisms of various structural changes is now beginning.

RETICULAR BASEMENT MEMBRANE (RBM)

A clearly defined change in asthmatics is thickening of the sub-epithelial RBM (22,28). The increased thickness of the RBM in asthmatic patients has been shown to be associated with increased bronchial hyper-reactivity (35,36), as well as with reduced FEV_1 (4) and an increased rate of exacerbations (36), but several studies have found no such correlations. Investigations of the correlation between RBM thickness and disease severity or AHR have also produced conflicting results (4,37–39). It has been suggested that activated eosinophils ($EG2^+$) are involved in the genesis of a thickened subepithelial reticular layer, through the production of cytokines such as transforming growth factor β (TGF-β) (37), but this remains to be validated.

An additional contribution to the remodelling of extracellular matrix in chronic airway inflammation stems from imbalances between enzymes that degrade matrix proteins (matrix metalloproteinases) and endogenous inhibitors of these enzymes (tissue inhibitors of metalloproteinases; TIMPs). One example is the breakdown of elastin by neutrophil elastase; greater activity of this enzyme has been reported in asthma patients and a negative correlation has been shown with FEV_1 (40).

Regulation of the activity of matrix metalloproteinase MMP-9 is similarly disrupted in asthma patients; expression of the enzyme is greater than that of its tissue inhibitor and has been associated with increased RBM thickness and reduced FEV_1 (41).

VASCULAR CHANGES

Increased blood flow to a site of injury and inflammation is a normal physiological response. Sustained pulmonary hyperaemia leads to an increase in the thickness of the bronchial wall which narrows the airway lumen (42,43). One study of autopsies and resected lung samples from asthma patients showed that the density of blood vessels in the airway wall was greater than that in control samples (44). Recent biopsy studies have demonstrated that asthma patients have more vascularized bronchial tissue than controls (45,46). A study of the vascularity of the lamina propria in asthma patients receiving inhaled corticosteroids (ICS) showed no difference in the density of vessels compared with patients not on ICS, although the vessel density in all patients was correlated with both AHR and post-bronchodilator FEV_1 (47).

SECRETION OF MUCUS

Hypersecretion of mucus into the airway lumen in asthmatic patients appears to be caused by both submucosal gland hypertrophy and goblet cell hyperplasia. Goblet cell hyperplasia is a prominent phenotypic change in the airway epithelium in newly diagnosed asthmatic patients (48). After 3 months of inhaled corticosteroid treatment, the epithelial structure was shown to be restored to normal ciliated epithelium, accompanied by a pronounced reduction in the number of inflammatory cells (13). One study has reported the proportion by volume of both mucin and goblet cells in the epithelium to be three times higher in asthmatic patients than in controls (49). This change may be driven by increased activity of platelet activating factor (PAF) or epidermal growth factor (EGF), both of which cause an increase in airway goblet cell number in animal models (50,51). EGF immunoreactivity in the epithelium, smooth muscle and goblet cells of autopsied bronchi from asthma patients has been shown to be greater than that from controls with no disease (52). IL-4 and IL-13 levels are also known to be raised in asthmatic patients (14) and both have been shown to stimulate goblet cell hyperplasia (53,54).

SMOOTH MUSCLE AND THE ROLE OF GROWTH FACTORS

Increased smooth muscle mass contributes to thickening of the airway wall. This has been demonstrated by comparing autopsy samples from asthma patients and controls with no respiratory disease (44,55,56) and by quantifying the effect of smooth muscle mass on bronchoconstrictor-provoked increases in airway resistance observed in asthma patients (57).

A role for so-called 'myofibroblasts' in remodelling was first proposed when such cells, defined by immunostaining, were shown to be associated with the thickening of the RBM and deposition of fibronectin in the bronchial mucosa of asthma patients (58). The number of myofibroblasts in the mucosa of asthmatic and bronchitic patients was found to be correlated with epithelial and submucosal expression of TGF-β produced by eosinophils and fibroblasts (59). Significant correlations have also been found between the increased number of myofibroblasts in asthma patients

compared with controls and the greater thickness of RBM containing collagen (types III and V) and tenascin (41).

Compared with controls, concentrations of TGF-β are raised in asthmatic patients before and 24 h after allergen challenge (60). The number of 'myofibroblasts' were also shown to be markedly increased in asthmatic patients following allergen challenge (61), but these cells are likely to be distinct from those associated with RBM thickening, since they showed ultrastructural similarity to bronchial smooth muscle and are suggested to represent de-differentiated smooth muscle of the synthetic phenotype. The changes seen after allergen challenge bear similarity to the alterations described in response to vascular injury and disease. Another growth factor which can be monitored in biopsies is GM-CSF. This cytokine may play a fundamental role in the early stage of remodelling by prolonging the survival of eosinophils in the airway of asthmatic patients (62).

Assessment of airway remodelling in clinical studies

The contribution of many processes to the phenomenon of airway remodelling in asthma patients complicates the selection of a single primary variable that would clearly signify the presence and rate of remodelling. Currently, the most favoured measurement is that of RBM thickness, but assessments of vascularity, myofibroblast numbers and phenotypic changes to airway and vascular smooth muscle by electron microscopy are becoming key research areas for the future.

Further studies on the structure of the RBM and its components in asthmatic patients and healthy controls are required to establish which components are significantly altered by the disease, and whether asthmatic patients have unique components not present in the healthy airway. Blood vessels from biopsy samples may be stained using markers of endothelium, e.g. CD31, or antibodies to type IV collagen which enable the basement membrane of the vessel wall to be visualized, so that the number of vessels per unit area and their diameter can be measured (45,46).

Other possible markers include those that measure the amounts of mucus-secreting tissue, i.e. the ratio of serous to mucous acini, gland size or goblet cell numbers in biopsy samples; analysis of mucin gene expression will also be important in the future (63,64). Once markers of remodelling have been clearly identified, the next challenge is to show that alterations in these markers are associated with changes in long-term clinical outcome.

Conclusion

The clinical presentation of asthma and the immunopathology of airway inflammation in asthma have been well characterized. The inflammatory mechanisms responsible for the involvement of T-cells, eosinophil infiltration. TH_2 cytokine expression and chemokine expression by inflammatory and structural cells can be studied directly in asthma patients by analysing bronchial biopsies. Variability of markers within the lung, airway wall and between patients, however, may reduce the reliability of estimates, and adequate sampling and careful application of quantitative technique are required. Possible distinctions between inflammation in atopic and non-atopic or in mild and severe asthma can be explored by employing specific markers such as HNL and MPO for neutrophils, and EG2 and ECP for eosinophils.

The field of chronic pulmonary inflammation is now being more intensively studied. Although the process of inappropriate airway remodelling is not yet understood, many of the contributing pathologies are now recognized and progress is beginning to be made in identifying potential markers that may be followed in long-term studies to discover more specific and effective treatments for chronic asthma in all its forms.

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